# SOME PURE CULTURE METHODS IN THE ALGÆ

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## INTRODUCTION

Too much confidence has frequently been placed by algologists in their ability to recognize a given species of alga among varying numbers of other species, and in the various forms which it may assume—a fact which has led to much confusion and error, especially among members of the *Protococcales*. While it is now definitely known that in a number of algæ a single species may present markedly dissimilar appearances, either as a result of varying environmental conditions, or because of the presence in the life history of several unlike stages, it is certain that much of the so-called *polymorphism*, or *pleomorphism*, of algæ finds its explanation in inadequate methods of study. It is becoming recognized that for life history studies in the algæ it is necessary to employ cultures free from other species of algæ. Even in cases where this is not, on first thought, necessary, as in the large, filamentous forms, it should be observed, for the possibility of introducing spores or sporelings of closely allied species is by no means excluded in all cases. Gratifying progress has already been made by some algologists, working especially with members of the *Volvocales* and *Protococcales*, and it seems reasonably certain that the originally chaotic condition existing in the latter will be ultimately reduced to complete order by a careful observance of the necessity of working with pure cultures, or at least cultures containing but a single species of alga. In life-history studies where physiological differences between species are to be investigated, it is especially desirable and indeed necessary to employ pure cultures.

Certain species of algæ, especially representatives of the *Chlorophyceæ*, have been much used in physiological investigations—chiefly those concerning themselves with various

phases of nutrition. With the development of a clearer understanding of the activities and life processes of the various micro-organisms, the necessity of working with rigorously pure cultures has become more and more evident. It is now generally appreciated that, in most cases, valid conclusions as to the physiology of a particular organism cannot be drawn with certainty where one or more foreign organisms have been present in the cultures. There can be no doubt that the frequent contamination of cultures of algæ with bacteria, and even with fungi, has, in many cases, detracted markedly from the value of painstaking and otherwise careful physiological investigations. The readiness, however, with which many algæ lend themselves to experimental purposes—on account of their small size and ease of handling and culture—will always make them favored objects of study; and it appears desirable at this time to bring together some of the experiences of the author in the preparation of pure cultures of algæ, with the hope that suggestions may be gained from them by those who desire to obtain such cultures for one purpose or another.

An unfortunate use of the term "pure culture" has come into more or less general use and has frequently led to confusion and ambiguity. As used by many authors, it means simply a culture of a single species of alga not necessarily free from bacteria and fungi. Where the presence of other organisms is not specifically mentioned, it is clear that the above usage of the term may lead to serious misunderstandings. Indeed, it remains for the reader, in many instances, to decide for himself—from the technique employed—whether a culture of an alga free from all other organisms or only from other species of algæ is meant. It is to be hoped, therefore, that the term pure culture shall come to have the same clearly defined meaning when used in connection with the algæ that it has long had in the fungi and bacteria. In the following report the term is used to signify a culture of a single species of alga free from all other organisms.

## HISTORICAL

Although incidental references to pure culture technique in the algæ are frequently found in the literature, relatively few contributions have appeared which deal extensively with the

subject, or which outline in detail the methods employed. Beyerinck, in 1890 (4, 6), appears to have been the first to succeed in isolating species of algæ in pure culture. Ditch water, boiled with ten per cent gelatin, and cooled, was mixed with a drop of water rich in protococcoid algæ, poured into dishes, and allowed to cool. Numerous minute algal colonies appeared in course of time, and the number of bacterial colonies developing was so small that successful transfers of *Scenedesmus acutus* Meyen and *Chlorella vulgaris* Bey., were made, both organisms being subsequently cultured on a variety of media. In addition, the gonidia of *Physcia parietina* were obtained pure. Small pieces of the lichen thallus, carefully washed, were placed on solid gelatin plates. Those which showed themselves to be free from foreign organisms were transferred to gelatin plates containing malt-extract, the fragment being first torn to bits with needles and then dragged over the sterile surface. In a few days, small colonies of the algal symbiont appeared from which successful transfers were made. In a later paper (5), Beyerinck adds *Stichococcus major* and a second species of *Chlorella* to the list of algæ previously cultured in a state of purity, the technique, in general, being the same.

Miquel (16) was the first to isolate a diatom in pure culture. Subsequently, Richter (20, 21) isolated *Nitzschia Palea* (Kütz.) W. Sm., and *Navicula minuscula* Grun., by the use of synthetic agar plates. Attention is called by this author to the importance of using agar which has previously been washed to free it from soluble impurities. A mixture of diatoms and other algæ was placed on the surface of washed agar plates, and from the impure diatom colonies which developed transfers were made to other plates until at length pure cultures were obtained.

In his isolations of certain protozoa in pure culture, Ogata (18) also obtained *Polytoma uvella.* While his method seems unnecessarily complex, it is of interest here. Sterile capillary tubes were filled in part with a column of sterile water, and subsequently a column containing the organisms was added below, care being taken not to separate the two by air. Both ends of the tube were then sealed. After sufficient time had elapsed for the movement of the motile *Polytoma* cells from the lower column into the upper sterile one, the tube was broken in

the region of the upper column. The lower portion was discarded, and the upper one was sealed, subsequently transferred to a sterile medium, and broken to permit the organisms, free from contaminations, to enter the medium and begin their development.

By the gelatin plate method, Krüger (13) prepared pure cultures of two new organisms—*Chlorella protothecoides* and *Chlorothecium saccharophilum*—obtained from the exudation of *Populus alba*. Tischutkin (23) lists representatives from about eighteen genera of algæ—including diatoms, green, and blue-green forms—as having been obtained in pure culture by the agar plate method. After three or four successive dilutions in liquid one per cent agar, the organisms were plated in Petri dishes. The filamentous forms he washed in sterile water, cut into short segments, and transferred to the liquid medium. The methods given by Ward (24) include plating in agar and silicic acid jelly, though as a whole the methods are applicable for the separation of algal species rather than for their isolation in pure culture. This is especially true of the plaster of Paris, and precipitated calcium carbonate methods. Gonidia from *Xanthoria parietina*, and *Gasparinia murorum* (Hoffm.) Tornab., together with *Pleurococcus vulgaris* and *Scenedesmus caudatus* were obtained in pure culture by Artari (1). Chodat and Goldflus (8), by the use of pieces of sterilized unglazed porcelain in contact with a mineral nutrient solution, claim to have isolated a species of *Nostoc* in pure culture. The procedure was a simple one, consisting in repeated transfers to fresh sterile plates until a pure culture was at length obtained.

Several years later Chodat and Grintzesco (9) reported that by essentially the same method, *Oocystis elliptica*, *Dictyosphærium pulchellum*, *Kirchneriella lunaris*, *Rhaphidium polymorphum*, *Pediastrum tetras*, *Scenedesmus acutus*, *Pleurococcus vulgaris*, *Hæmatococcus lacustris*, and *Chlorella vulgaris* had been obtained in pure culture. In cases where the number of algal individuals is small, but the bacteria and fungi relatively abundant, the authors point out the desirability of first increasing the number of the former by introducing the mixture into a mineral nutrient solution favorable for the growth of the algæ but not so for the fungi. Where filamentous forms are

concerned, the authors state that it is necessary to begin with the zoöspore, as a pure culture from filaments is extremely difficult to obtain. My own experience does not bear out this statement in all cases as it was found that especially among the *Ulotrichales* pure colonies were regularly and easily obtained from filaments.

Artari in 1902 (2) reports the isolation of *Chlorococcum infusionum* and *Scenedesmus caudatus* in pure culture. Chick (7) attempted to isolate *Chlorella pyrenoidosa* through the use of sterilizing agents such as hydrogen peroxide and sunlight. These trials, however, did not prove successful, as the alga failed to show a resistance sufficiently greater than that of the bacteria to make possible a successful separation. The isolation was finally attained by placing a few drops of water containing the organism on a sterile synthetic agar plate, and spreading the same over the surface with a brush. The same brush was used to distribute sterile water drops over the surface of other plates, no additional algal material being added. From the later dilutions pure colonies were obtained. Frank (10) was unable to obtain pure cultures of *Chlamydomonas tingens* by the agar plate method.

Jacobsen (11) reports the isolation of *Chlorogonium* and *Polytoma* in pure culture. This author made use of an interesting method of separation of algal species based on their different degrees of resistance to drying. Discs of filter paper, on which drops of water containing *Spondylomorum* and *Chlamydomonas variabilis* had been placed, were dried in an incubator at 28°C. After twenty-four hours, the discs were placed in a suitable medium, but only the *Chlamydomonas* species developed, *Spondylomorum* having been killed. *Chlorogonium euchlorum* and *Polytoma uvella* also showed themselves very sensitive to drying, whereas *Chlamydomonas* usually survived the desiccation. Old cultures of *Chlorogonium euchlorum* proved to be very resistant owing to the presence of zygospores which had been formed by the conjugation of gametes.

While reference might be made to a number of other investigations which deal in an incidental way with pure culture technique, it is believed that those given will serve to indicate, in a general way, the present status of the subject. (For further

information the reader is referred to Moore (17), Richter (21), Küster (14), and others.) It is apparent that the large majority of forms isolated in pure culture belong to the *Protococcales*. Only a few of the filamentous forms, several diatoms, and but one or two species of the blue-green algæ have thus far yielded to pure culture technique.

## Pure Culture Technique

### General

Algæ, generally speaking, are provided with a more or less highly developed exterior mucilaginous investment which may be either a distinct, separable sheath, as in many of the *Cyanophyceæ*, or merely a gelatinization resulting either from a modification of the external portion of the membrane, or from an internal secretion, as in some of the desmids. In general, also, algæ are slow growing as compared with many fungi. In these two characteristics most of the difficulties encountered in pure culture technique among the algæ find their explanation.

Among the fungi, spores with non-gelatinous walls are readily obtainable in a majority of the forms, and usually in great abundance. When such spores are plated in the way ordinarily employed in bacteriological technique, a large number of colonies free from bacteria are usually obtained. Among the algæ, however, such non-gelatinous, resistant spores are, if produced at all, generally present only in small quantities. When vegetative algal cells are plated on a suitable medium, algal colonies will often be obtained, but they usually form the nucleus of a larger bacterial colony which has developed from the bacteria adhering to the gelatinous surface of the algal cell. Among those fungi in which spores are not readily obtained, an isolation in pure culture may frequently be effected by allowing the fungus to grow on a suitable medium until the hyphæ have outstripped the bacteria in their growth, at which time pure mycelial transfers may be made from the terminal portions. If, however, a like procedure is attempted with the algæ it will usually be found that the bacteria adhere tenaciously to the surface of the growing filaments and are carried

along by the lengthening filaments. Except in rare cases, nothing is to be gained by this procedure in the algæ. The task of isolating pure cultures of algæ, therefore, becomes an individual problem for almost every species as it necessitates at once the determination of the period in the life history of any form at which the cells are free from bacteria or at which time the bacteria can be removed by one means or another. Having found a stage in which the alga is bacteria-free, it is of importance next to be able to bring about this stage more or less at will in order that the alga may be utilized when available. To obtain the above preliminary information, nothing is more serviceable than the usual plating method on a suitable medium.

*The Medium.*—The requirements of a suitable solid medium for algal isolating purposes are, that it remain liquid down to a temperature at which delicate algal cells are not injured; that it be suitable for the growth of algæ, and as unfavorable as possible for the growth of bacteria and fungi. For this purpose nothing was found so serviceable as the following, the mineral ingredients being in the proportions recommended by Moore (17):

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|---|---|
| Agar | 10.0 grams |
| $NH_4NO_3$ | 0.5 gram |
| $MgSO_4 . 7H_2O$ | 0.2 gram |
| $K_2HPO_4$ | 0.2 gram |
| $CaCl_2$ | 0.1 gram |
| $FeSO_4$ | trace |
| Dist. $H_2O$ | 1000 cc. |

The agar should be carefully washed, first in a stream of tap water and then in distilled water, as pointed out by Richter (20). An agar so prepared will remain liquid down to about 34.5–35°C., and experience has shown that even the most delicate algal cells are uninjured by the short exposure to this temperature necessary in the plating process. From six to eight cc. of agar in a Petri dish eight cm. in diameter is a suitable quantity with which to plate. Larger quantities so thicken the layer of agar in the dish that the higher powers of the microscope, with their objectives of short focal length, cannot be used in locating small developing colonies.

*Material to be Plated.*—The alga to be plated should be collected with as little adhering foreign matter as possible. If it is a filamentous form which can be manipulated with a platinum needle, it can be materially cleansed by washing in sterilized nutrient solution such as is used in the preparation of the agar. If the alga is a unicellular form, little can be done in the way of preliminary cleansing. Dilutions are made in the usual manner, the degree depending upon the number of algal organisms present. The degree of dilution will depend in part, also, upon the number of bacteria and fungi present as determined by microscopic examination. It must be remembered that the algæ grow more slowly than most bacteria and fungi, and that unless the dilution, from the standpoint of the total number of organisms present, is great enough, the spread of bacterial and fungal colonies may be so great as to make the transfer of the later-appearing algal colonies impossible without contamination.

The material should be introduced into the tube of liquid agar while the latter is still a few degrees above its congealing point, in order that the inoculated tube may be vigorously shaken for some time before its contents are poured into the Petri dish. In this way the algal cells are freed of large numbers of either accidentally or regularly adhering bacteria.

*Incubation and Transference.*—The plates, after the agar has solidified, should be turned upside down in order to prevent the moisture which condenses on the cover from dropping, and spreading bacteria over the surface of the agar. Failure to do this often renders large numbers of platings worthless. The most favorable place to keep plates is in the light of a north window; and, as plates frequently remain under observation for many weeks, it is further desirable to have them in a glass case to prevent outside contamination. In general it is not advisable to cover the plates with bell jars, as it increases the humidity in the Petri dishes and accelerates the growth of moulds present as contaminations. The plates should be examined frequently and when rapidly spreading colonies of fungi or bacteria appear, these should be dissected out in order to save the remainder of the plate.

The length of time necessary for the appearance of the algal

colonies varies greatly with the species, from one to three or four weeks usually being required, depending upon the particular form. In most cases it is not possible to wait until the algal colonies can be seen macroscopically because spreading bacterial and fungal colonies usually encroach on the former to such an extent that a pure transfer is no longer possible. It becomes necessary, therefore, to look the plates over from time to time with the compound microscope in order to locate algal colonies in very early stages of development. For this purpose a 12 mm. objective is extremely serviceable, as its focal length is of sufficient magnitude to enable one to use it through the agar layer and glass bottom of a Petri dish and at the same time obtain a magnification considerably greater than that afforded by the ordinary low-power objective. The colonies located are conveniently marked by placing a small ink dot directly opposite them on the bottom of the Petri dish. Transfers should be made to agar slants by means of a minute platinum-foil spatula with which the agar directly over the ink dot can be neatly dissected out and transferred to the slant. It is not possible, in most cases, to make successful transfers with a platinum needle because the algal colony is usually composed of firmly cohering cells and, even in repeated attempts, not a single individual will adhere to the needle. Since many of the colonies are in the deeper strata, it is well to spread out the transferred agar fragment in a thin sheet in order to expose the contained algal cells directly to the air. Unless this is done, subsequent development in the slant may be extremely slow. Although bacteria grow slowly on this synthetic agar, their development is usually sufficient in a week to indicate whether the transfer has been successful or not. The purity of the culture may be further tested by making transfers to media more suitable for bacterial growth.

With this brief preliminary consideration of some of the more general phases of pure culture technique in the algæ, the isolation of single species will now be considered and attention called to the special problems and the technique involved in their isolation.

SPECIFIC

CHLOROPHYCEÆ

*Chlamydomonas pisiformis* Dill forma *minor* Spargo.—*Chlamydomonas* species frequently occur in water rich in organic materials, and teeming with bacteria. When the alga was in the resting condition, the mucilaginous cell walls were found so impregnated with bacteria as to render isolation in pure culture impossible. Platings with motile cells, however, showed that the latter were absolutely free from regularly adhering bacteria, but the number of bacteria present rendered the plates worthless. Then the gelatinous masses of resting cells were repeatedly washed with sterile water and finally placed in distilled water where, after twelve to twenty-four hours, zoöspores appeared in great abundance and congregated on the side of the vessel nearest to the light. A minute portion of this liquid containing the zoöspores was removed with a fine capillary tube and introduced into a tube of liquid agar and plated. In platings thus made, numerous colonies of *Chlamydomonas* appeared and the number of bacterial colonies was so small that a large number of successful pure transfers were made.

Where the number of available motile cells is small and it is important that isolations be made from these, a modification of the method used by Barber (3) in the isolation of yeasts and bacteria was frequently used to advantage. A large number of small, capillary pipettes were made and sterilized. After locating the cell or cells desired, they were removed with a pipette while being observed under the microscope, and transferred to a drop of sterile nutrient solution or water. This process was repeated until it was certain that the number of bacteria had been reduced sufficiently to admit of successful plating. They were then taken up again by means of a sterile pipette, transferred to a tube of liquid agar, and plated. Numerous pure cultures were obtained in this way.

*Stichococcus bacillaris* Näg., and *S. subtilis* (Kütz.) Klercker.—Preliminary platings with these forms showed that the cells, as obtained from the soil, yielded abundant bacteria-free colonies, and the problem of isolation became one of merely obtaining clean material and diluting sufficiently. Both of these species

of *Stichococcus* are soil-inhabiting and can be obtained—practically free from other algæ—on flower pots and greenhouse soils. The former species, because of its minute cells and the readiness with which the filaments resolve themselves completely into their constituent cells when placed in water, is a particularly easy one to obtain in pure culture. Rich material may be diluted until plates obtained from it show a sufficiently small number of bacterial colonies to admit of pure transfers and yet enough algal colonies for a number of transfers. *S. subtilis* is a larger species and the cells remain attached in rather long filaments. However, with vigorous shaking and previous teasing apart with needles, a sufficient number of single cells and small fragments of filaments are introduced to make possible numerous successful isolations. The washing of the cells to remove adhering bacteria can, in these species and many others, be largely accomplished by introducing the raw material into test-tubes containing sterile mineral nutrient solution or water, stoppering, and shaking vigorously. Direct transfers from these to liquid agar, or to tubes of sterile water for further dilution, may then be made. This procedure frequently enables one to make successful platings where the direct transfer of raw material to liquid agar results in constant failure.

*Chlorella vulgaris* Bey., and *Chlorella sp.*—Both of these species were isolated from soil in the open. An exterior gelatinous investment is, as in the two above mentioned species of *Stichococcus*, conspicuously absent, and preliminary experiments demonstrated that a large number of the vegetative cells were freed from all accidentally adhering bacteria by being shaken in the liquid agar before plating. The problem of isolating these species again becomes one of clean material and sufficient dilution. Species of *Chlorella* are perhaps the easiest among the algæ to isolate in pure culture, the process requiring little more than a direct application of bacteriological methods.

Attention should be called to another method—really a modification of the one just given—by means of which *Chlorella* species may be obtained in pure culture. Its application is not necessary in the species of *Chlorella* investigated, since

the vegetative cells can be so readily freed from adhering bacteria. But its general applicability to other forms justifies its mention at this place. *Chlorella*, like many other genera of the *Protococcales*, forms non-motile endogenous daughter cells which remain enclosed in the mother wall for varying lengths of time. The enclosed daughter cells are in all cases free from adhering bacteria. A group of daughter cells still enclosed within the mother membrane may be removed by means of a capillary pipette to a drop of sterile water, and from here to a succession of others until all readily removable bacteria have been left behind. The last transfer should be made to a drop of sterile water on a small sterile cover glass. By a slight pressure of a second cover glass, the mother membrane may be ruptured, liberating the enclosed, bacteria-free cells. The two cover glasses should then be introduced into a tube of liquid agar, the latter shaken vigorously, and finally poured into a Petri dish. Frequent isolations have been made in this way, and its importance in forms whose vegetative cells cannot be freed from adhering bacteria, and which do not form motile spores but only non-motile endogenous daughter cells, can hardly be overestimated.

*Pleurococcus vulgaris* Menegh.—The majority of *Pleurococcus* cells, when thoroughly washed, will be found free from bacteria. A difficulty which frequently arises is that the alga grows so very slowly that fungi—which are persistently present in *Pleurococcus* cultures—take entire possession of the plates before a transfer can be effected. But with careful searching, minute colonies—often consisting of but a few cells—can usually be found and successfully transferred. The transferred colony, however, usually makes extremely slow progress in its growth on agar. Much better results are obtained when transfers are made to evaporimeters (as devised by Livingston (15)) supplied with the mineral nutrient solution.

*Scenedesmus sp.*, and *Kirchneriella sp.*—Both of these species were obtained in pure culture by washing and diluting clean, concentrated material in sterile mineral nutrient solution, and then plating. The great majority of the colonies of both species were contaminated with bacteria, pure colonies being very rarely found. This fact, together with the gelatin-

ous exterior characteristic of the cells of both species, makes it probable that the pure colonies developed, not from mature individuals, but from autocolonies (produced within mature cells) which either had just escaped from the mother cell or had done so during the vigorous shaking,—in either of which cases they are free from adhering bacteria.

*Chlorococcum humicola* (Näg.) Rabenh.—This species was isolated in the zoösporic condition. The alga, collected from soil, was placed in sterile mineral nutrient solution and after twenty-four hours produced zoöspores in abundance. Platings with these yielded numerous pure colonies from which successful transfers were made. In this connection it should be mentioned that all zoöspores thus far experimented with—including a considerable variety of forms—have been found free from bacteria. It is needless to say, therefore, that the presence of zoöspores in the life cycle of any alga provides a logical point of attack for its isolation in pure culture. While not all the attempts to isolate zoösporic forms in pure culture have proved successful, it is entirely probable that they will when the general technique is more closely adapted to individual forms.

*Protosiphon botryoides* (Kütz.) Klebs.—The vegetative plant of *Protosiphon*, with its root-like process extending into the soil and the large aerial portion, is so persistently covered with bacteria that its isolation in pure culture in this condition is quite impossible. With slight desiccation, however, large numbers of chlamydospores with dry non-gelatinous membranes appear, which, at least so long as they remain enclosed within the mother membrane, are free from bacteria. From these, isolations in pure culture can be readily made according to the second method suggested for *Chlorella*—by carefully washing an individual plant filled with chlamydospores, liberating the latter by teasing with needles or by a slight pressure of the cover glass, and plating in the usual manner. Another method which has yielded pure cultures, but which is not to be recommended because it is far less reliable than the one just described, is based on the use of the motile gametes. When vigorous *Protosiphon* plants, growing on soil, are covered with distilled water, gametes, which congregate in the lighted

side of the vessel, are produced in large numbers. Plates made with this material yield an occasional pure culture, but most of the gametes fail to develop. It is impossible at present to say whether the colonies develop from newly formed zygotes or from gametes which fail to conjugate.

*Stigeoclonium tenue* (Ag.) Kützing.—The ease and certainty with which zoöspores can be induced to develop in this form, and their extreme abundance, makes it, although a filamentous alga, an especially easy one to isolate. Freshly collected and thoroughly washed filaments of *Stigeoclonium*, placed in distilled water or sterile nutrient solution, will, in from twelve to twenty-four hours, develop a great abundance of zoöspores. Cultures prepared in this way contain so small a number of bacteria that plates containing a hundred or more *Stigeoclonium* zoöspores are sufficiently free from bacterial colonies to render numerous successful pure transfers possible. Although a filamentous form, *Stigeoclonium* grows exceedingly well on the mineral nutrient agar. While other members of the *Chætophoraceæ* were not experimented with, it is reasonably certain that forms like *Microthamnion*, *Chætophora*, and *Draparnaldia*, all of which readily yield large quantities of zoöspores, may be obtained in pure culture by a method identical with or similar to the one employed in the isolation of *Stigeoclonium*.

*Oedogonium sp.*, and *Vaucheria sp.*—While neither of these forms were obtained in pure culture, the observations made render it altogether likely that this will be possible when a little more attention is given to the cultural solutions. Repeated trials with the vegetative filaments demonstrated that from the latter no pure cultures could be obtained directly. The oöspore proved equally unsatisfactory because the oögonial wall is covered with adhering bacteria. Again, the oöspore is, in most cases, so firmly and completely united with the oögonial wall that its separation from the latter is at present impossible. In both forms, however, zoöspores are readily obtained, and preliminary experiments demonstrated that these, like zoöspores in general, are bacteria-free. Where zoöspores could not be obtained in large quantities, individual ones were isolated with sterile pipettes, washed repeatedly in sterile water, and then either plated in the usual manner, or introduced into a

tube of sterile mineral nutrient solution. Although the great majority of such isolations remained bacteria-free, the zoöspores failed to develop, and finally died. It is only necessary, therefore, to find some medium in or on which the zoöspores will germinate and develop into plants, to effect a pure culture of *Vaucheria* or *Oedogonium*. *Bulbochæte* was not used, but in all probability this form will lend itself to a similar technique.

*Conjugales.*—Thus far it has not been possible to obtain a pure culture of any member of the *Conjugales*. The representatives of this order, in their vegetative phases, are provided throughout with an exterior gelatinous investment which is very generally impregnated with bacteria. All attempts to obtain pure cultures from vegetative individuals failed. Further, there is a complete absence in the order of motile spores and, in general also, of separable, asexual, endogenous spores. The zygospore, therefore, suggests itself as a possible means of solving the problem, especially in those forms where it is produced endogenously, and where it does not subsequently coalesce with the wall of the gametangium. While pure cultures were not obtained from these, the method used in *Spirogyra setiformis* is of interest and may prove serviceable in the ultimate isolation of these forms in pure culture.

Filaments containing mature zygospores, but in which the zygospore-containing cell walls were still completely intact, were washed repeatedly in sterile water and then broken up as thoroughly as possible with needles; in this process, numerous zygospores were freed from the enclosing walls, later to be taken up with sterile pipettes, and transferred to sterile drops of water. Each zygospore was subsequently transferred from ten to twenty times to fresh, sterile water drops, and finally taken up with a sterile pipette. When a considerable number of zygospores had thus been isolated, they were introduced into a tube containing a few cc. of sterile water, vigorously shaken, and the entire contents poured out into a Petri dish containing a layer of sterile nutrient agar. After rocking the dish for a short time, it was allowed to remain quiet until the zygospores had settled down on the surface of the agar. The free water was then very slowly and carefully, but completely, drained from the surface of the agar, and the plate allowed to remain

in the light. While in a few cases bacterial colonies developed about the zygopsores, it was found that the great majority were free from all adhering bacteria. Such zygospores as were bacteria free were then transferred to test tubes containing sterilized mud and pond water. Although about sixty such transfers were made, not a single one yielded a growing culture, although zygospores kept in battery jars in the laboratory showed a high percentage of germination. It will require further experiments to find a suitable medium for the germination and subsequent growth of isolated zygospores. However, the isolation of bacteria-free zygospores justifies the opinion that with them it will, sooner or later, be possible to culture *Spirogyra* in a state of purity.

HETEROKONTÆ

*Botrydium granulatum* (L.) Greville.—This form is, in its general morphology, so similar to *Protosiphon*, that the technique, as regards the use of chlamydospores, described for the latter, is entirely applicable here. *Botrydium* when submerged, however, forms an abundance of zoöspores instead of gametes, and from these pure cultures can be obtained with great ease when plated in the usual manner. The method for using the chlamydospores can also be considerably abbreviated in *Botrydium*. When the plants form chlamydospores, the aerial globular portion of the plant collapses. The cell, however, is so large that the aerial bag can be torn open with fine sterile forceps, the spores removed under a hand lens with a needle and transferred directly to liquid agar. Platings made in this way show a very slight bacterial contamination, and pure transfers can be made in abundance. While a direct, bacteria-free transfer has not been thus effected, it is altogether probable that it can be done. The pure transfers of *Botrydium* having been obtained, it was found that their development on agar was extremely slow, and ultimately all of the cultures died. Further experiments will be necessary in order to provide a favorable medium for growth. The clay-cup evaporimeter may perhaps prove of service in this connection as it did in the case of *Pleurococcus*.

*Botrydiopsis sp.*—This form was found abundantly during

one season on soil in the greenhouses. The vegetative cells when placed in water readily produce zoöspores, and isolations were made from these with little difficulty. Unlike *Botrydium*, this form grows exceedingly well on the mineral nutrient agar.

### BACILLARIALES

The diatoms were encountered only incidentally in connection with other forms, and no particular effort was made to isolate forms in pure culture. Although diatoms, in general, have a gelatinous exterior, a small *Navicula* was on several occasions obtained in pure culture and grown successfully. It should be said, however, that the great majority of diatom colonies obtained were contaminated with bacteria.

### CYANOPHYCEÆ

In the class *Cyanophyceæ*, the most difficult problems of isolation are met. The almost universal presence of an abundance of external mucilaginous material, the complete absence of ciliated reproductive cells, and the virtually complete absence of free, endogenous spores, renders the technique particularly difficult. The gelatinous investments are, in all cases investigated, impregnated with bacteria which cannot be completely removed by the most vigorous washing. Among the forms studied were *Aphanocapsa*, several species each of *Oscillatoria*, *Nostoc*, and *Anabœna*, *Cylindrospermum*, and *Microcoleus*. Of these, only two species, one of *Oscillatoria* and one of *Microcoleus*, were obtained in pure culture.

In the isolation of these two forms, silicic acid jelly was found to be indispensable. While directions for preparing this medium are to be found in many places in the literature, certain difficulties encountered in its preparation have made it desirable to give at this time, and in some detail, the method used.

As regards the preparation and mixing of the sodium silicate and hydrochloric acid solutions, the directions given by Smith (22) may be followed. It is only necessary to point out in this connection that if Merck's "sodium silicate pure crystals" is used, the solution should be made up with cold water. If hot water is used, an unidentified substance (insoluble in cold water) goes into solution, and frequently causes the coagulation

of the silicic acid-hydrochloric acid mixture before dialysis is complete. A point of very great importance is the preparation of the collodion dialyzing bags. As has been pointed out by Kellerman (12), and others, the degree of permeability of the bags depends, in a large degree, upon the way in which they are made. If the guncotton solvent is made from equal parts of ether and absolute alcohol, the bags will, in most cases, have a very low permeability, and coagulation of the enclosed silicic acid solution will frequently result before dialysis is complete. The degree of impermeability is further increased by drying the bags rapidly. If, however, 95 per cent (instead of absolute) alcohol is used, and the bags are allowed to dry spontaneously by inverting the test-tubes in which the bags are being prepared in suspended wire baskets, a much higher degree of permeability will be obtained.

Bags prepared with 95 per cent alcohol were used, and the silicic acid-hydrochloric acid mixture dialyzed in tap water until the chloride content was no greater than that of the water. The silicic acid solution was further purified by dialyzing in changes of ordinary distilled water and finally in triply distilled, nitrogen-free water. In this extended dialysis, a considerable portion of the silicic acid is lost, and it usually becomes necessary to concentrate the solution to obtain a jelly of sufficient firmness. This is best carried out in heavy, two-liter suction-flasks in which the pressure is reduced until the solution boils at from 35 to 40°C. If the concentration is carried out at higher temperatures, coagulation sometimes results. In order to prevent the violent bumping which always takes place unless some special precautions are taken, it is only necessary to bring through the rubber stopper at the top of the suction-flask a glass tube drawn out at the bottom to a very fine capillary, which dips into the solution. The top of this tube, outside of the rubber stopper, should be provided with a piece of rubber tubing and pinch cock to regulate the intake of air. The air thus admitted may first be washed to remove carbon dioxide, ammonia, or other impurities. The concentration should be continued until a sample, when congealed, has the proper consistency. The directions given by Smith (22) for coagulating the medium apply here and it need only be mentioned

that the concentration of the mineral nutrients employed in the agar, 0.1 per cent, is quite sufficient to bring about coagulation.

After it had become probable that no blue-green alga, in the ordinary vegetative condition, could be isolated by the usual plating method, tubes containing from two to three inches of solid, sterile, synthetic agar were inoculated at the surface with a species of *Oscillatoria*. The tubes were then completely wrapped in black paper, leaving only the very bottom exposed to the light, and inverted. It was hoped that in the rapid growth of the alga through the agar, the bacteria might be left behind. The growth toward the light in some cases amounted to eight mm., and more, per day. When the growth had approximately reached the bottom of the tube, the end of the latter was broken away, the surface of the agar seared, and transfers made from the interior of the agar plug. Although the experiment was repeated many times, and a total of at least fifty transfers made, a pure culture was never obtained, bacteria always being present. Large Petri dishes, containing a layer of sterile synthetic agar, were then inoculated at one edge with a species of *Oscillatoria*, and the dishes so placed that the point of inoculation was farthest away from the light. The alga grew rapidly (on the surface of the agar) toward the light, and just before reaching the opposite edge of the dish, transfers were made from the farthest advanced filaments. Although transfers to fresh agar surfaces were continued to the number of six, a pure culture was never obtained.

The experiment was then repeated, surfaces of silicic acid jelly replacing those of agar, with the result that numerous pure transfers were obtained from the second plate. A species of *Microcoleus* was obtained in pure culture in an identical manner.

Most members of the *Oscillatoriaceæ* are provided with a sharply delimited, gelatinous sheath. Reproduction is effected by the formation of hormogonia which glide out of the sheath, move about slowly for a time, and then come to rest. In forms like *Microcoleus*, *Lyngbya*, and some species of *Oscillatoria* in which the hormogonia escape from definite sheaths,

leaving the latter behind, it is fairly certain that the hormogonium is originally free from bacteria, but becomes contaminated in passing through the older portion of the empty sheath and out of its terminal opening, both of which are more or less infected with bacteria. The persistence with which the bacteria cling to the hormogonium of *Oscillatoria*, once having infected it, is clearly shown by cultures on agar surfaces. Although a single hormogonium may have moved as much as two inches away from its parent filament, creeping all the while over a sterile agar surface, the hormogonium will be found covered with bacteria, and the path over which it moved will be clearly indicated by a continuous, linear colony of bacteria. With the use of silicic acid jelly, however, the multiplication of the bacteria is reduced to such an extent that, after a time, hormogonia escape uncontaminated, and begin the development of pure colonies. Transfers from these, however, grow very slowly and in most cases eventually die. It seems probable, when *Oscillatoria* and *Microcoleus* have been completely separated from the invariably present bacteria, that the media which were favorable in the presence of the bacteria, become unfavorable in their absence. Further work will be necessary to grow these forms successfully after they have been isolated in pure form. The silicic acid jelly method was also attempted with the above mentioned heterocystic forms; however, up to the present time, no successful isolations have been made.

## DISCUSSION

It is apparent that the technique involved in the isolations just referred to depends entirely on mechanical separation of one kind or another. This method is reasonably efficient in those species in which zoöspores or other free endogenous spores are readily obtainable, or in which vegetative cells are either free from bacteria or can be rendered so by mechanical means. It is true that even in some species forming free endogenous spores, the above methods have not yielded pure cultures, as, for instance, in *Vaucheria*, *Oedogonium*, and *Spirogyra*. In these cases, however, it should be pointed out that it is not the isolation technique which is at fault but rather

the cultural methods. Zoöspores and zygospores, respectively, free from other organisms, were obtained in these cases but failed to develop in the cultural media subsequently supplied. There can be little doubt, however, that the latter difficulty will be overcome in time.

Except in the *Oscillatoriaceæ*, little progress was made in the *Cyanophyceæ*. The problem appears especially difficult in the *Coccogoneales* where all forms of motile reproductive bodies are absent, and in which the vegetative cells apparently cannot be rendered free from adhering organisms by mechanlica means. Even in the heterocystic *Hormogoneales*, the situation is a difficult one, the more slowly moving hormogonia apparently being unable to escape the bacteria.[1] While no experiments were made along these lines, it appears highly desirable to attack the problem in the latter group through the spore. It is well known that the spores of blue-green algæ are extremely resistant to heat, and it does not appear improbable that the bacteria—especially if they are all in the vegetative condition—could be killed by heat, leaving the algal spores unharmed. Chemical sterilizing agents may also prove of value here. The latter may also prove serviceable with members of the *Coccogoneales* and certain of the grass-green algæ which have thus far failed to yield to the technique employed.

## Conclusions

1. By adapting methods of pure culture technique to individual species of algæ, it has been possible to isolate in pure culture the following forms:

*Chlorophyceæ.—Chlamydomonas pisiformis* Dill forma *minor* Spargo, *Stichococcus bacillaris* Näg., *S. subtilis* (Kütz.) Klercker, *Ulothrix sp.*, *Chlorella vulgaris* Bey., *Chlorella sp.*, *Pleurococcus vulgaris*, *Scenedesmus sp.*, *Kirchneriella sp.*, *Chlorococcum humicola* (Näg.) Rabenh., *Protosiphon botryoides* (Kütz.) Klebs, *Stigeoclonium tenue* (Ag.) Kützing, and a number of others of uncertain identity.

[1] In a contribution which has just appeared (Kulturversuche mit Chlorophyll-führenden Mikroorganismen, III. Zur Physiologie der Schizophyceen. Beitr. z. Biol. d. Pflanzen 12: 49–108. 1913), Ernest G. Pringsheim reports the isolation in pure culture of a species of *Nostoc*. The method used was that of repeated transfers to sterile silicic acid jelly plates.

*Heterokontæ.—Botrydium granulatum* (L.) Greville, and *Botrydiopsis sp.*

*Bacillariales.—Navicula sp.*

*Cyanophyceæ—Oscillatoria sp.*, and *Microcoleus sp.*

2. In addition, zoöspores from *Vaucheria* and *Oedogonium*, and zygospores from *Spirogyra* have been isolated free from other organisms.

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